Journal of proteome research

pubs.acs.org/jpr

Technical Note

Comparative Analysis of Lysine-Specific Peptidases for Optimizing Proteomics Workflows

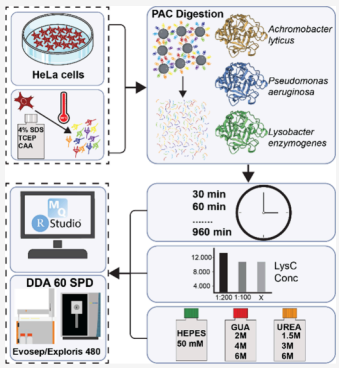
Leander Roeland van der Hoeven, Maico Lechner, Cristina Hernandez-Rollan, Tanveer Singh Batth, and Jesper V. Olsen*

Cite This: *J. Proteome Res.* 2026, 25, 1176−1183

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: This study presents a comparative analysis of three LysC endopeptidase homologues from *Achromobacter lyticus* (*A. lyticus*),*Pseudomonas aeruginosa* and *Lysobacter enzymogenes* for mass spectrometry-based proteomics. Utilizing a protein aggregation capture workflow with HeLa cell lysates, we assessed the enzymes' cleavage specificity, digestion efficiency, and performance across various experimental conditions. Results showed that while all three LysC homologues exhibited high cleavage specificity at lysine residues, *A. lyticus* LysC outperformed the two others with superior peptide identification, digestion efficiency, and protein coverage, especially at shorter digestion times. Our experiments using a combination of*A. lyticus*LysC and trypsin demonstrated the importance of employing LysC for significantly minimizing missed cleavage rates in tryptic digests, especially with regard to lysine-containing peptides. This study underscores *A. lyticus* LysC's potential as an optimal choice for enhancing mass spectrometry-based proteomics.

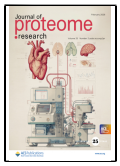


KEYWORDS: *endoproteinase LysC, proteolytic enzymes, proteomics workflow, label-free quantitation, method development, LC-MS/MS, bottom-up proteomics*

INTRODUCTION

Mass spectrometry (MS) is a pivotal analytical technique extensively utilized in proteomics research for the identification and quantification of proteins and their modifications. It is employed in both targeted and exploratory proteomics to analyze proteins and peptides from various biological sources, such as cells, tissues, and fluids like blood or urine.[1] This technology is also increasingly applied in fields like drug development, food safety, and environmental analysis.[2] The core strength of MS lies in its ability to provide precise, sensitive, and reproducible measurements of biomolecular compositions.[3] A typical proteomics workflow involves several stages, including protein digestion into peptides, which are amenable to liquid chromatography−tandem mass spectrometric (LC-MS/MS) analysis.[4] The complexity of these workflows necessitates meticulous handling and the use of highly sequence-specific enzymes to minimize technical variability to ensure reproducibility and quantitative results. Lysine-specific endopeptidases (LysC) are essential tools in proteomics workflows, enabling efficient digestion of proteins into smaller peptides for MS-based identification and quantification.[5] LysC is a serine protease that specifically cleaves peptide bonds at the carboxyl side of lysine residues and is commonly used in combination with trypsin, a serine peptidase that cleaves the C-terminus to arginines and lysines. Unlike trypsin, LysC is more resistant to denaturants such as urea[6] and guanidinium[7] and can also cleave lysine residues adjacent to proline.[8] Therefore, LysC is often used to predigest protein mixtures under denaturing conditions, whereby proteins are unfolded, allowing LysC to access and cleave lysine residues more effectively. This step is crucial for ensuring a more comprehensive breakdown of protein structures and digesting proteolytically resistant proteins effectively before diluting the denaturants prior to the addition of trypsin. This combination of peptidases is used to enhance digestion efficiency and reduce missed cleavages at lysine residues.[7] While the LysC−trypsin combination is currently the gold standard for most MS-based workflows, the performance of different LysC homologues has not been directly compared. Additionally, the properties and efficiency of LysC under different conditions have not been evaluated. LysC was initially discovered in 1970 in the bacterium *Lysobacter enzymogenes* (*L. enzymogenes*) but was soon identified in other bacterial species, including *Achromobacter lyticus* (*A. lyticus*) and*Pseudomonas aeruginosa*(*P. aeruginosa*).[6,9,10] Efficient protein digestion by proteases such as LysC is influenced by several factors, including the choice of enzyme, sample preparation, and digestion conditions, such as pH,

Received: September 29, 2025
Revised: December 2, 2025
Accepted: December 11, 2025
Published: December 22, 2025



https://doi.org/10.1021/acs.jproteome.5c00872
J. Proteome Res. 2026, 25, 1176−1183

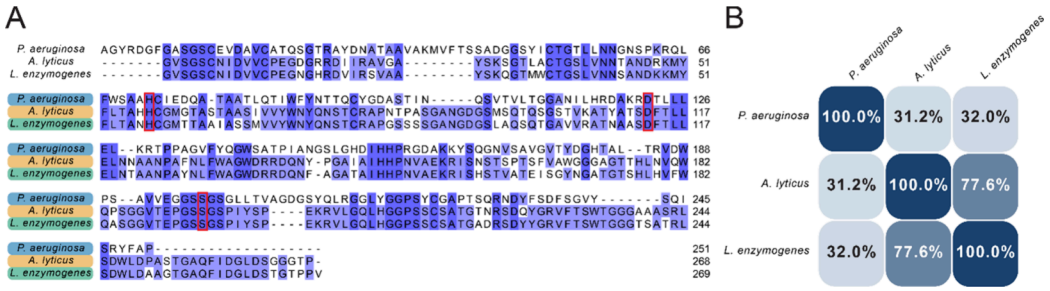


Figure 1. Sequence analysis of the three LysC enzymes used and workflow representation of the study. (A) Left panel: sequence alignment of the catalytic regions of LysC enzymes from *P. aeruginosa*, *A. lyticus*, and *L. enzymogenes*. Conserved regions are highlighted in blue; the conserved catalytic triad regions essential for activity are highlighted in red. (B) Homology score in percentage between the three LysC enzymes.

temperature, and choice of buffers, among others. However, specific digestion properties, such as the efficiency of different LysC protein homologues under varying conditions, have not been directly compared. Therefore, a deep dive could not only improve robustness but also help improve conditions for best practices in peptide quantitation. While these homologues share some similarities in amino acid composition, especially *L. enzymogenes* and *A. lyticus*, as we demonstrate, they differ in some of their digestion properties. The structural differences among the LysC homologues are crucial in defining their biochemical properties and interactions with substrates and inhibitors, ultimately impacting their functionality and application in various biotechnological contexts. However, to date, there has not been a comprehensive study comparing these homologues and their impact on protein digestion in MS-based experiments. In summary, this study underscores the importance of optimizing both LysC selection and digestion conditions to improve proteomics workflows. Among the homologues tested, *A. lyticus* LysC yielded the highest peptide identifications and superior cleavage efficiency. An optimized LysC−trypsin workflow is therefore recommended for comprehensive, reproducible MS-based proteomics analysis.

MATERIALS AND METHODS

Materials

LysC sequencing-grade recombinant enzymes from *Achromobacter lyticus* (KPL0033), *Lysobacter enzymogenes* (*KPL0013*), and *Pseudomonas aeruginosa* (*KPL0022*) were kindly provided from KPL ApS, Copenhagen, Denmark.

Cell Culture

Immortalized human epithelial cervix carcinoma adherent cells (HeLa) were grown in 500 cm^2 dishes in DMEM (Gibco, Waltham, MA, USA) media containing 2 mM L-glutamine supplemented with 10% fetal bovine serum (Gibco, Waltham, MA, USA), 100 U/mL penicillin (Life Technologies, Carlsbad, CA, USA), and 100 μg/mL streptomycin in a 37 °C incubator supplemented with 5% CO_2.

Sample Preparation

Cells were lysed with 1 mL of boiling lysis buffer (4% SDS; 100 mM Tris pH 8.5, 5 mM TCEP, and 10 mM CAA) on a plate and scraped, followed by incubation at 95 °C, for 10 min with mixing (1000 rpm). Lysates were sonicated with a tip probe (3 min, 3 s on, 2 s off, 100% amplitude, Fisherbrand probes model 120 sonic dismembrator). The protein concentration was calculated using the BCA assay and tryptophan assay.[11,12] For all experiments, 10 μg of protein lysates was digested per sample. Digestion was done following the protein aggregation capture protocol as previously described by Batth et al.[13] Briefly, proteins were aggregated on magnetic microbeads (MagResyn Hydroxyl beads) by adding acetonitrile (ACN) to a final concentration of 70%. Beads were washed once with 100% ACN and once with 70% ethanol. 50 mM HEPES digestion buffer at pH 8.5 was added to all samples prior to the addition of proteases. Unless stated otherwise, a typical protease-to-protein ratio of 1:100 was employed for all of the digestion. The digestion reaction was quenched by adding trifluoroacetic acid (TFA) to a final concentration of 1%. Thereafter, an equivalent to 500 ng of the digestion was loaded into Evotips for subsequent MS analysis.[14]

LC-MS/MS Analysis

Digested peptides were separated on an EV-1109 column (PepSep, 8 cm × 150 μm, beads 1.5 μm) and an EV-1087 emitter (fused silica, 20 μm) with an Evosep One LC system. The column was interfaced online using an EASY-Spray source with an Orbitrap Exploris 480 MS (Thermo Fisher Scientific, Bremen, Germany) using Xcalibur (tune version 4.0 or higher). In all samples, the spray voltage was set to 1.8 kV, the funnel RF level was set at 40, and the capillary temperature was heated to 275 °C. All experiments were acquired using a data-dependent acquisition (DDA) top 20 method with a 60-samples-per-day (SPD) LC gradient.

Data Analysis

All Thermo raw files were searched with the MaxQuant software suite 2.4.13.0 at default settings or LysC/P semi-specific search using a human database (UniProt reference proteome 2023 release, 20,598 entries) in addition to a contaminant FASTA file containing porcine-trypsin and the LysC homologues utilized in this study when required.

The output files were analyzed using RStudio with the following packages: dplyr (1.1.2), readr (2.1.5), tidyr (1.3.1), ggplot2 (3.5.1), ggrepel (0.9.3), stringr (0.9.3), ggfortify (0.4.17), ggpattern (1.1.1), biomaRt (2.56.1), httr (1.4.7), ggseqlogo (0.2), and jsonlite (1.8.8).

RESULTS

To identify the best LysC for proteomics research, we analyzed the cleavage specificity and digestion efficiency of three LysC homologues from *L. enzymogenes*, *A. lyticus*, and *P. aeruginosa*. The three LysC homologues are the primary LysC enzymes used for proteomics. Of note is that all the experiments were performed using a workflow based on protein aggregation capture (PAC).

https://doi.org/10.1021/acs.jproteome.5c00872
J. Proteome Res. 2026, 25, 1176−1183

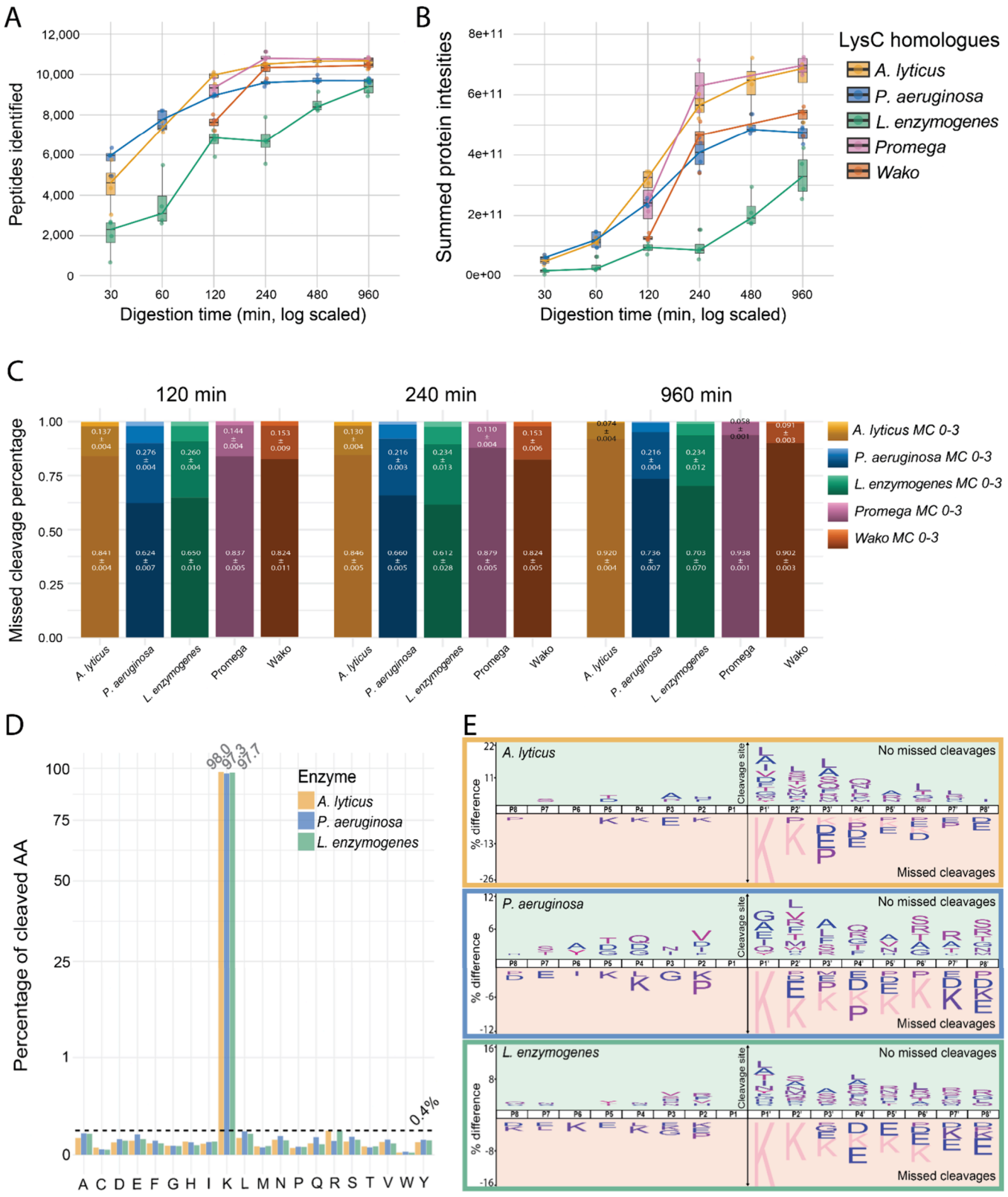


Figure 2. Digestion efficiency evaluation of the three LysC homologues. (A) Comparison of the number of identified peptides (B) and of the total peptide intensity over a digestion time course ranging from 30 to 960 min for the three LysC homologues. (C) Cleavage efficiency of the three LysC homologues measured by the percentage of missed cleavages. (D) Catalytic specificity evaluation for the three LysC homologues. (E) Motif analysis of the 3 lysC homologues around the cleavage site.

A protein sequence alignment of the LysC enzymes from*A. lyticus*,*P. aeruginosa*,and*L. enzymogenes*reveals conserved regions essential for enzymatic function, especially the catalytic triad that is essential for activity. The triad residues consist of serine, histidine, and aspartic acid; for*A. lyticus*and*L. enzymogenes*LysC, these can be found at Ser194, His57, and Asp113; for*P.*

https://doi.org/10.1021/acs.jproteome.5c00872
J. Proteome Res. 2026, 25, 1176−1183

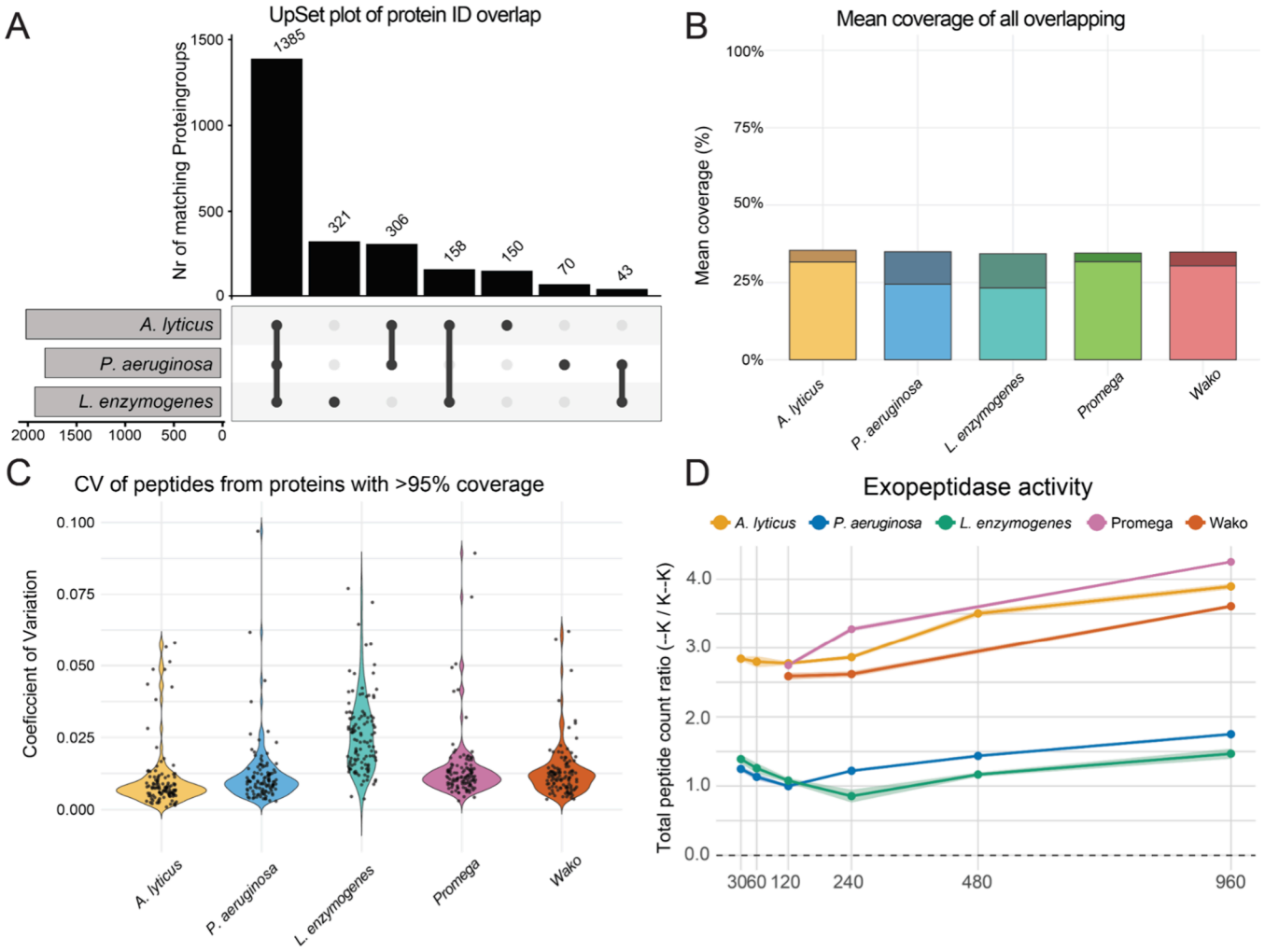


Figure 3. Comparison of protein identification, digestion patterns, reproducibility, and exopeptidase activity across different LysC enzymes. (A) UpSet plot of proteins identified by each LysC enzyme. (B) Protein coverage analysis of the top 20 proteins by total coverage, comparing the digestion patterns of the three LysC homologues and the commercial enzymes. Here, the mean coverage of the top 20 proteins is shown by fully cleaved peptides compared to coverage by peptides containing at least 1 missed cleavage. (C) CV values of peptides from proteins with a coverage of at least 95%. (D) Line plot showing the ratio between peptides containing 1 vs >1 N-terminal lysine residues over differing time points.

aeruginosa, the positions for these residues are Ser198, His72, and Asp122 (excluding the propeptide and signal peptide sequences) (Figure 1A,B). Despite the higher sequence homology between*A. lyticus* and*L. enzymogenes*than with*P. aeruginosa*, this did not translate into similar functional performance; as shown later in this study,*A. lyticus*and*P. aeruginosa*instead displayed more comparable functional behavior in the context of proteomics workflows. To benchmark the performance of these three enzymes against each other, we applied them in an experimental sample preparation workflow using HeLa cell lysates and subsequent on-bead digestion, ranging from 30 to 960 min. The resulting peptide mixtures were then analyzed by online LC-MS/MS. Each digestion condition was analyzed in four technical replicates to assess reproducibility.

As an initial benchmark, we assessed the three LysC homologues alongside the two commercial enzymes (Promega and Wako) by plotting the total number of HeLa peptides identified across the digestion time course. This revealed that LysC from*A. lyticus* consistently identified more peptides, especially at early time points (Figure 2A and Figure S1A), indicating superior performance in complex samples, especially when compared to*L. enzymogenes*. This trend was also observed when analyzing the summed total peptide intensities as a proxy for protease efficiency (Figure 2B and Figure S1A). The trend was largely the same when evaluating the three LysC homologues based on the total HeLa protein numbers identified as a function of digestion time, with the exception that with the overnight digestion, they all identified an almost comparable number of proteins (Figure S1A). To assess if the observed difference in protease efficiency is linked to their cleavage activity, we analyzed this as a function of digestion time. Cleavage efficiency represented by the percentage of peptides with missed lysine cleavage sites confirmed that*A. lyticus* exhibits the highest enzymatic efficiency with the lowest level of missed cleavages observed, reaching close to 90% efficiency at early time points and 92% of detected peptides with zero missed cleavages after an overnight digestion (Figure 2C and Figure S1A). Conversely, LysC homologues from both *P. aeruginosa*and*L. enzymogenes*achieved about 70% cleavage efficiency at best (Figure 2C and Figure S1A).

To evaluate the quantitative performance of the three LysC homologues, we analyzed the coefficient of variation (CV) values for the identified peptides across the four replicates.

https://doi.org/10.1021/acs.jproteome.5c00872
J. Proteome Res. 2026, 25, 1176−1183

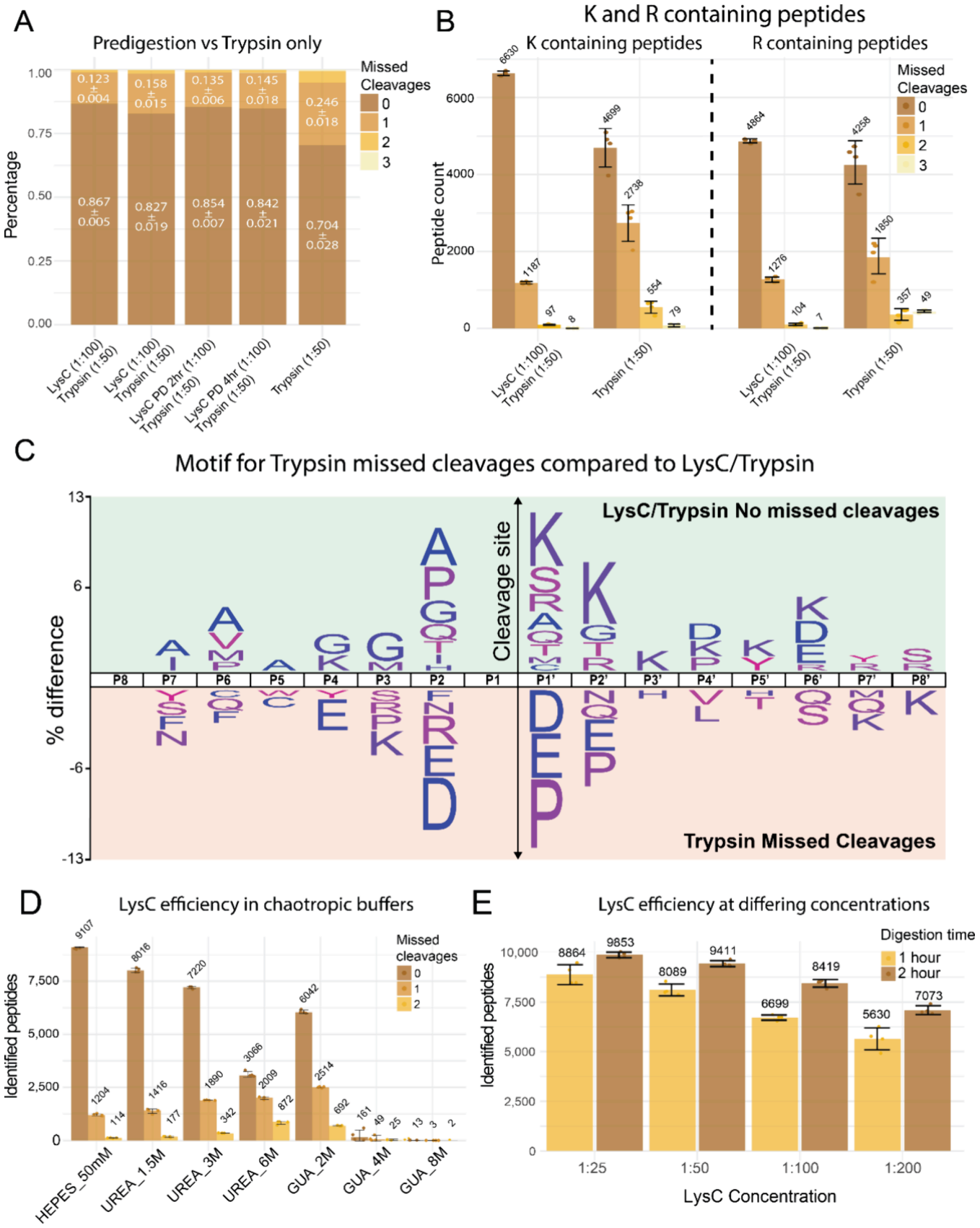


Figure 4. Evaluation of LysC digestion efficiency and cleavage specificity of *A. lyticus* across varying conditions. (A) Comparison of LysC concentrations and digestion methods for the *A. lyticus* LysC. (B) Number of peptides cleaved at K or R sites, comparing the combined LysC–trypsin digestion with trypsin alone. (C) Motif of peptides with 0 missed cleavages in the LysC/trypsin combination and at least one missed cleavage in trypsin-only digests. (D) Digestion efficiency of *A. lyticus* under different denaturing buffers. (E) Digestion efficiency of *A. lyticus* at several concentrations and time points.

https://doi.org/10.1021/acs.jproteome.5c00872
J. Proteome Res. 2026, 25, 1176−1183

This analysis showed that*A. lyticus*and*P. aeruginosa* display similar CV values, while*L. enzymogenes* shows higher and more variable CVs, indicating more consistent performance over time for both*A. lyticus and P. aeruginosa*(Figure S1B). Finally, we examined the number of peptides identified per protein for each of the LysC enzyme variants. As shown in Figure S2A, none of the LysC variants show an increase in the average number of peptides identified per protein (Figure S2A).

To evaluate the cleavage specificity of the three LysC homologues, we reanalyzed the overnight digestion data using MaxQuant, allowing for semispecific peptide matches. Cleavage specificity analysis of all identified peptide sequences showed that all three LysC homologues are highly specific, with more than 97% of all cleavages at the C-terminal of lysine residues and no apparent secondary preferences (Figure 2D). To identify whether any sequence bias near lysine residues might contribute to missed cleavages, we performed a sequence motif enrichment using Icelogo.[15] This approach can highlight sequence-specific patterns contributing to missed cleavages, suggesting structural features affecting enzyme−substrate interactions. The analysis revealed an enriched sequence pattern for missed cleavage lysine sites with bias against proline (P), glutamic acid (E), and additional lysine (K) residues in close proximity to the cleavage site situated at the P1 residue (Figure 2E). This enriched motif was highly similar for all three LysC homologues; however, some differences were observed. Although a reduction in cleavage efficiency due to a proline on the +1 position was observed for all three LysC homologues, *P. aeruginosa* seems to be especially susceptible to this effect. Additionally,*A. lyticus* is hardly affected by the proximity of lysines near the cleavage site.

To compare the HeLa proteins identified by the three LysC homologues, we first examined their overlap. An UpSet plot showed the shared and unique proteins of each LysC homologue (Figure 3A).*A. lyticus* identifies the most unique proteins, underscoring its enhanced coverage. Additionally, the commercial LysC enzymes from Wako and Promega were also analyzed, which showed very similar performance to the *A. lyticus* homologue (Figure S2B). To examine digestion patterns, we compared the median sequence coverage for all of the overlapping proteins (Figure 3B and Figure S3A,B) as well as for two additional selected proteins (Figure S3C). Of the three recombinantly made LysC homologues, *A. lyticus* yielded the highest fraction of fully cleaved peptide coverage (∼30%) versus*P. aeruginosa*and*L. enzymogenes*that cover ∼25% of proteins by fully cleaved peptides. The two commercially available LysC enzymes from Wako and Promega, respectively, performed similarly to*A. lyticus*. For proteins with ≥95% sequence coverage, peptide-level CVs were lowest for*A. lyticus* and highest (in addition to the most dispersed) for*L. enzymogenes*, with Promega and Wako LysC again closely matching*A. lyticus* (Figure 3C). Given that*A. lyticus* reduced sensitivity to proximal lysines and its more complete cleavage, we hypothesized a higher exopeptidase activity. Consistent with this, across the time course, *A. lyticus* produced a higher ratio of peptides and an increased intensity of these peptides, with a single N-terminal lysine relative to those with >1 N-terminal lysines (Figure 3D and Figure S3D), comparable to the Promega and Wako enzymes.

To assess potential biases in protein classes identified by the three LysC homologues, we grouped the identified proteins by their subcellular localization based on data from the DeepLoc 2.0 project.[16] Unsupervised hierarchical clustering of log10-transformed intensities across four replicates per enzyme was visualized in a heat map (Figure S4A,B). The heat map exhibited distinct patterns of protein abundance across different subcellular components. The hierarchical clustering revealed that the replicates of each LysC homologue group together, indicating high consistency within each LysC homologue. The clustering on the subcellular localization axis showed two subgroups, emphasizing a difference between proteins localized in the nucleus and cytoplasm. The second cluster includes the subcellular components of the mitochondrion, endoplasmic reticulum, cell membrane, and extracellular proteins. Intensities indicated that LysC from *A. lyticus* showed the most similarities with *P. aeruginosa*. *L. enzymogenes* showed overall lower protein intensities for all localizations compared to the two other LysC enzymes, which was coherent with the overall reduced performance of *L. enzymogenes* displayed in Figure 2A. To further evaluate if there is statistical significance between the three LysC homologues, we performed a one-way ANOVA statistical test for each subcellular localization group comparing the sum of squares (SS), mean squares (MS), and degrees of freedom (df) between and within each group (Figure S4A). Based on this, input *F*-values were calculated and compared to the tabulated critical *F*-values. The results are presented as *p*-values, indicating a statistically significant difference between the variance of a localization if the *p*-values are below a significance threshold of 0.05. The *F*-values and corresponding *p*-values indicated significant differences across all localizations, with the highest *F*-value observed in the mitochondrion (77.8, $p = 2.09 \times 10^{-6}$), suggesting a substantial variation of the intensities between the three LysC homologues. The endoplasmic reticulum and cytoplasm also showed high *F*-values (46.3 and 48.3, respectively), indicating significant differences. The cell membrane and nucleus exhibited lower but still significant *F*-values (17.7 and 13.5, respectively) (Figure S4B). The sum of squares between groups is notably high in the cytoplasm and nucleus, reflecting substantial variability in these regions. The mean squares between groups are consistently higher than those within groups, reinforcing the significant impact of the different LysC homologues and suggesting a good reproducibility between technical replicates. All the above experiments described in Figures 1−4 consistently demonstrated *A. lyticus* as the superior LysC homologue compared to the two others.

As the most frequent use of LysC in proteomics is in combination with trypsin, we next tested the impact of combining *A. lyticus* LysC with sequencing-grade modified trypsin for overnight digestion using a fixed trypsin-to-protein ratio of 1:50. LysC was used in different protease-to-protein ratios either employing a predigestion step prior to trypsin addition or used simultaneously in combination with trypsin. The impact of LysC addition was evaluated based on the fraction of missed cleavage rates at arginines and lysines and compared to trypsin-only digestion (Figure 4A). A LysC predigestion step showed that the optimal protease combination is a LysC-to-protein ratio of 1:100 together with a trypsin-to-protein ratio of 1:50 (Figure 4A). However, predigestion of the protein mixture with LysC did not seem to have an effect on reducing the number of missed cleaved peptides compared to adding LysC and trypsin at the same time. All tested combinations showed a comparable ∼85% identification of tryptic peptides with zero missed cleavages (Figure 4A and Figure S5A). Conversely, using trypsin without LysC significantly increases the number of missed cleavages with

https://doi.org/10.1021/acs.jproteome.5c00872
J. Proteome Res. 2026, 25, 1176−1183

only ∼70% identification of tryptic peptides with zero missed cleavages. This is especially apparent in the lysine-containing peptides where ∼30% of all identified peptides contain a missed cleavage when using trypsin without LysC, whereas less of a difference is observed for arginines (Figure 4B and Figure S5B). This analysis reveals that peptides containing lysines are more prone to missed cleavages during tryptic digests, suggesting that trypsin is suboptimal for cleaving lysines compared to arginines. This demonstrates the importance of using LysC in combination with trypsin for minimizing missed cleavage sites in tryptic digests. From a motif analysis, it can be observed that trypsin shows reduced cleavage efficiency when proline is present at the P1′ position compared to LysC, as is well-known in the literature.[17] Additionally, trypsin cleavage efficiency is affected by the presence of aspartic acid (D) and glutamic acid (E) at position P1′ (Figure 4C).

As LysC is frequently used to predigest protein mixtures under denaturing conditions harsher than those trypsin can tolerate, we next tested the cleavage efficiency of *A. lyticus* LysC in different denaturing buffers. This analysis showed that, when digesting a HeLa lysate at varying concentrations of denaturing buffers, the performance of LysC remains relatively stable in urea up to 1.5 M (Figure 4D). At higher urea concentrations, a similar number of peptides are identified, however, with a markedly increased number of missed cleavages. Conversely, LysC is more sensitive to guanidinium (GUA), showing a decrease in the number of identified peptides, even at 2 M GUA. At the highest concentration tested, peptide identification drops close to zero (Figure 4D).

Finally, to identify the best compromise between the LysC-to-protein ratio and digestion time, we analyzed different combinations of digestion time and LysC concentration. When digesting with a higher concentration of *A. lyticus* LysC, the digestion time showed an almost linear curve compared to the LysC concentration (Figure 4E). Interestingly, this analysis suggests that as a rule of thumb, doubling the amount of LysC leads to the same number of peptides identified in half the digestion time.

The comprehensive benchmark analysis of three LysC homologues from *A. lyticus*, *P. aeruginosa*, and *L. enzymogenes* provides valuable insights into their applicability for mass spectrometry-based proteomics research. This study evaluated the enzymes' cleavage specificity, digestion efficiency, and overall performance. The results consistently demonstrated that LysC from *A. lyticus* outperformed its counterparts in terms of peptide identification and digestion efficiency. Particularly with short digestion times of a few hours, this suggested its potential to enhance the sample throughput of proteomics studies, especially when a higher LysC endopeptidase to protein ratio was chosen. This superior performance was further evidenced by its high cleavage efficiency, reaching nearly 90% at early time points and 92% after overnight digestion with the lowest level of missed cleavages observed among the three LysC homologues. In proteins with ≥95% sequence coverage, peptide-level CVs were lowest for *A. lyticus* and highest, with the most dispersed distribution, for *L. enzymogenes*, while the commercial LysC enzymes (Promega and Wako) closely matched *A. lyticus*. In terms of quantitative performance, *A. lyticus* and *P. aeruginosa* LysCs displayed more consistent reproducibility compared to *L. enzymogenes* as measured by CV values across replicates. This consistency is crucial for quantitative proteomics, in which reproducibility is paramount. All three LysC homologues exhibited high specificity for lysine residues, with over 97% of cleavages occurring at the C-terminal of lysine.

Moreover, a sequence motif analysis revealed a bias against glutamic acid and additional lysine residues near cleavage sites in missed cleavages, which could be valuable for predicting potential missed cleavages and optimizing digestion protocols. Additionally, it was shown that LysC is affected by proximal prolines. However, when compared to trypsin, the performance of LysC in the presence of proximal prolines is markedly better than that of trypsin. In protein coverage analyses, *A. lyticus* achieved the highest fraction of fully cleaved peptide coverage versus *P. aeruginosa* and *L. enzymogenes*, and the commercial enzymes performed similarly to *A. lyticus*, with Promega showing slightly higher fully cleaved coverage. Consistent with reduced sensitivity to proximal lysines and more complete cleavage, *A. lyticus* showed a higher ratio of peptides with a single N-terminal lysine relative to those with >1 N-terminal lysines across the time course, comparable to Promega and Wako. The subcellular localization analysis revealed distinct patterns among the LysC homologues, with *A. lyticus* showing a broader distribution of proteins across cellular compartments, particularly in mitochondria and cytoplasm. Investigating the use of LysC in combination with trypsin at different protein-to-protease ratios and predigestion steps provided valuable insights for optimizing proteomics workflows. Trypsin digestion without LysC yielded significantly higher missed cleavage rates, particularly at lysine residues, emphasizing the importance of combining LysC together with trypsin for enhancing tryptic digestion. The study found that the direct combination of LysC (1:100) and trypsin (1:50) yielded optimal results without the need for a predigestion step, potentially streamlining sample preparation protocols. Additionally, the study showed the stability of *A. lyticus* LysC in varying concentrations of denaturing buffers, offering flexibility in protein extraction and digestion buffers, particularly when dealing with challenging protein samples. While this study provides a comprehensive comparison of the three most popular LysC homologues, several avenues for future research emerge. These include structural analysis to understand the molecular basis for varying efficiencies, evaluation of performance across diverse sample types, exploration of potential synergistic effects with other proteases, and application in large-scale, quantitative proteomics studies to assess its impact on proteome coverage in real-world applications.

In conclusion, this study provides compelling evidence for the superior performance of *A. lyticus* LysC in proteomics workflows. Its high efficiency, specificity, and consistency make it an excellent choice for researchers who aim to enhance the depth and quality of their proteomics analyses. The insights gained from this comparative study not only inform the selection of proteases for current research but also pave the way for further optimization and innovation in proteomics methodologies.

■ ASSOCIATED CONTENT

Data Availability Statement

The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD068827.

https://doi.org/10.1021/acs.jproteome.5c00872
J. Proteome Res. 2026, 25, 1176−1183

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.5c00872.

(Figure S1) Digestion efficiency evaluation and CV evaluation of the three LysC homologues, (Figure S2) evaluation of the number of peptides per protein and overlap of identified proteins, (Figure S3) protein coverage of protein by peptides identified with the different LysC homologues in addition to exopeptidase activity by peptide intensity, (Figure S4) distribution of identified proteins to different compartments in the cell to determine specific cleavage specificity for these types of proteins, and (Figure S5) comparison of LysC from *A. lyticus* and trypsin digestion efficiency and variability in peptide identification (PDF)

AUTHOR INFORMATION

Corresponding Author

Jesper V. Olsen − *Novo Nordisk Foundation Center for Protein Research, Department of Cellular and Molecular Medicine, University of Copenhagen, Copenhagen N DK-2200, Denmark;* orcid.org/0000-0002-4747-4938; Email: jesper.olsen@cpr.ku.dk

Authors

Leander Roeland van der Hoeven − *Novo Nordisk Foundation Center for Protein Research, Department of Cellular and Molecular Medicine, University of Copenhagen, Copenhagen N DK-2200, Denmark*

Maico Lechner − *Novo Nordisk Foundation Center for Protein Research, Department of Cellular and Molecular Medicine, University of Copenhagen, Copenhagen N DK-2200, Denmark*

Cristina Hernandez-Rollan − *Novo Nordisk Foundation Center for Protein Research, Department of Cellular and Molecular Medicine, University of Copenhagen, Copenhagen N DK-2200, Denmark; KPL ApS, Copenhagen 2200, Denmark;* orcid.org/0000-0002-4818-3630

Tanveer Singh Batth − *Novo Nordisk Foundation Center for Protein Research, Department of Cellular and Molecular Medicine, University of Copenhagen, Copenhagen N DK-2200, Denmark; KPL ApS, Copenhagen 2200, Denmark*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jproteome.5c00872

Notes

The authors declare the following competing financial interest(s): Jesper V. Olsen, Tanveer S. Batth and Cristina Hernandez-Rollan are co-founders of KPL ApS, Copenhagen, Denmark.

ACKNOWLEDGMENTS

The LysC sequencing-grade recombinant enzymes used were kindly provided from KPL ApS, Copenhagen, Denmark. Work at The Novo Nordisk Foundation Center for Protein Research (CPR) is funded in part by a donation from the Novo Nordisk Foundation (NNF14CC0001, NNF21OC0072070, and NNF24SA0098829). This work has been funded by Independent Research Fund Denmark|Natural Sciences under grant agreement no. 2032-00311B. This project was supported by a center-of-excellence grant from the Danish National Research Foundation to Copenhagen Center for Glycocalyx Research (DNRF196). The project was also supported by a generous grant from the Danish Agency of Higher Education and Science to establish the PLATO research infrastructure: Danish National Mass Spectrometry Platform for Proteomics and Biomolecular Imaging (grant no. 5229-00012B). J.V.O. and M.L. are also funded by Novo Nordisk a/s (CELFFI-2022-002843).

REFERENCES

(1) Aebersold, R.; Mann, M. Mass spectrometry-based proteomics. *Nature* **2003**, *422* (6928), 198−207.

(2) Dettmer, K.; Aronov, P. A.; Hammock, B. D. Mass spectrometry-based metabolomics. *Mass Spectrom. Rev.* **2007**, *26* (1), 51−78.

(3) Yates, J. R.; Ruse, C. I.; Nakorchevsky, A. Proteomics by mass spectrometry: approaches, advances, and applications. *Annu. Rev. Biomed. Eng.* **2009**, *11*, 49−79.

(4) Zhang, Y.; Fonslow, B. R.; Shan, B.; Baek, M. C.; Yates, J. R. Protein analysis by shotgun/bottom-up proteomics. *Chem. Rev.* **2013**, *113* (4), 2343−2394.

(5) Giansanti, P.; Tsiatsiani, L.; Low, T. Y.; Heck, A. J. Six alternative proteases for mass spectrometry−based proteomics beyond trypsin. *Nat. Protoc.* **2016**, *11* (5), 993−1006.

(6) Glatter, T.; Ludwig, C.; Ahrné, E.; Aebersold, R.; Heck, A. J.; Schmidt, A. Large-scale quantitative assessment of different in-solution protein digestion protocols reveals superior cleavage efficiency of tandem Lys-C/trypsin proteolysis over trypsin digestion. *J. Proteome Res.* **2012**, *11* (11), 5145−5156.

(7) Jekel, P. A.; Weijer, W. J.; Beintema, J. J. Use of endoproteinase Lys-C from Lysobacter enzymogenes in protein sequence analysis. *Anal. Biochem.* **1983**, *134* (2), 347−354.

(8) Gershon, P. D. Cleaved and missed sites for trypsin, Lys-C, and Lys-N can be predicted with high confidence on the basis of sequence context. *J. Proteome Res.* **2014**, *13* (2), 702−709.

(9) MASAKI, T.; NAKAMURA, K.; ISONO, M.; SOEJIMA, M. A new proteolytic enzyme from Achromobacter lyticus M497−1. *Agric. Biol. Chem.* **1978**, *42* (8), 1443−1445.

(10) Cahan, R.; Axelrad, I.; Safrin, M.; Ohman, D. E.; Kessler, E. A secreted aminopeptidase of Pseudomonas aeruginosa. Identification, primary structure, and relationship to other aminopeptidases. *J. Biol. Chem.* **2001**, *276* (47), 43645−43652.

(11) Wiśniewski, J. R.; Gaugaz, F. Z. Fast and sensitive total protein and peptide assays for proteomic analysis. *Anal. Chem.* **2015**, *87* (8), 4110−4116.

(12) Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **1985**, *150* (1), 76−85.

(13) Batth, T. S.; Tollenaere, M. X.; Rüther, P.; Gonzalez-Franquesa, A.; Prabhakar, B. S.; Bekker-Jensen, S.; Deshmukh, A. S.; Olsen, J. V. Protein Aggregation Capture on Microparticles Enables Multipurpose Proteomics Sample Preparation. *Mol. Cell. Proteom.* **2019**, *18* (5), 1027a−1035.

(14) Bache, N.; Geyer, P. E.; Bekker-Jensen, D. B.; Hoerning, O.; Falkenby, L.; Treit, P. V.; Doll, S.; Paron, I.; Müller, J. B.; Meier, F.; Olsen, J. V.; Vorm, O.; Mann, M. A Novel LC System Embeds Analytes in Pre-formed Gradients for Rapid, Ultra-robust Proteomics. *Mol. Cell. Proteom.* **2018**, *17* (11), 2284−2296.

(15) Colaert, N.; Helsens, K.; Martens, L.; Vandekerckhove, J.; Gevaert, K. Improved visualization of protein consensus sequences by iceLogo. *Nat. Methods* **2009**, *6* (11), 786−787.

(16) Ødum, M. T.; Teufel, F.; Thumuluri, V.; Almagro Armenteros, J. J.; Johansen, A. R.; Winther, O.; Nielsen, H. DeepLoc 2.1: multi-label membrane protein type prediction using protein language models. *Nucleic Acids Res.* **2024**, *52* (W1), W215−W314.

(17) Keil, B. *Specificity of Proteolysis*; Springer-Verlag Berlin Heidelberg. (1992). .